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(54) **VECTEUR POUR UNE THERAPIE GENIQUE SPECIFIQUE DU FOIE**

(54) **VECTOR FOR LIVER-SPECIFIC GENE THERAPY**

(57) Vecteur spécifique à des tissus et suffisamment spécifique au foie pour la thérapie génique d'un hôte malade, où la maladie est, comme il se doit, une hépatopathie, comprenant un virus d'insecte qui contient (i) une séquence d'ADN thérapeutique, (ii) un promoteur favorisant la transcription génique dans le tissu cible, comme il se doit le tissu hépatique de l'hôte, et, de façon facultative, (iii) une séquence d'établissement et des procédés de préparation et d'administration à l'hôte.

(57) A tissue specific, suitably liver specific vector for gene therapy of a diseased host, wherein the disease is suitably a diseased liver, comprising an insect virus that contains (i) a therapeutic DNA-sequence, (ii) a promoter for gene expression in the target tissue, suitably the liver tissue of the host, and optionally (iii) an establishment sequence, and processes for its preparation and administration to the host.

Abstract of the disclosure

A tissue specific, suitably liver specific vector for gene therapy of a diseased host, wherein the disease is suitably a diseased liver, comprising an insect virus that contains (*i*) a therapeutic DNA-sequence, (*ii*) a promoter for gene expression in the target tissue, suitably the liver tissue of the host, and optionally (*iii*) an establishment sequence, and processes for its preparation and administration to the host.

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VECTOR FOR LIVER-SPECIFIC GENE THERAPY

Field of the invention

The invention relates to a vector for tissue-specific, suitably liver-specific gene therapy. It is particularly useful in medicine, and in genetic engineering.

Background of the invention

Numerous methods and vectors have been developed in recent years for gene therapy. See, e.g. the survey by Mulligan, in Science, vol. 260, p. 926. Many vectors are favored in this connection for gene therapy, particularly all those which are derived from retroviruses or from adenoviruses. Both types of viral vectors are relatively broadly useful, retroviral vectors being generally effective only in proliferating cells. Adenoviruses also infect nondividing cells. Although both types of vectors are suitable for gene transfer *in vitro* into liver cells (hepatocytes), they can hardly be considered for use in *in vivo* gene therapy application in humans. Although a partial liver resection is necessary to stimulate cell division (regeneration) in the application of retroviral vectors, the adenoviral

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gene transfer is not very stable because no gene integration into the genome takes place.

Alternative vectors with potential applicability for gene transfer to liver cells are based on liposomes or also on multicomponent particles with protein domains, which bind specifically to certain receptors of the liver, such as the asialoglycoprotein receptor and can be taken up in the cell due to receptor internalization. These were surveyed by Versland et al. in 1992, Seminars in Liver Disease 12, 332. An important disadvantage of these vectors is the endocytotic absorption pathway, which leads to a degration of a large portion of the vectors and their DNA in the endosomes, so that only little DNA capable of functioning, can reach the cell nucleus.

A solution to this problem was found for the *in vitro* applications, but that is not useful for *in vivo* use in patients. The principle is based on the simultaneous infection of the target cells with adenovirus, which leads to a disruption of the endosomes and a release of vector (DNA), as described by Curiel, D.T., Agrawal, S., Wagner, E. and Cotten, M. 1991, PNAS 88, 8850 - 8854.

It was proposed in our German patent application No. 4,339,922.3 to pack a therapeutic gene, which is coupled to a promoter and is able to correct a genetic defect for the disease that is to be treated, in a peptide coat and to couple it to a component of the hepatitis B (HBV) or other specific virus, thus fulfilling an important prerequisite for treating genetic diseases of the liver or other tissue.

Description of the drawing

The invention is explained also with reference to the sole drawing, showing 10^4 cells lysed in 50 ml of lysis buffer (100 mM KH_2PO_4/K_2HPO_4, pH 7.8, 1% Triton X-100;* 1mMDTT) and measured in 180mℓ reaction buffer (25 mM KH_2PO_4/K_2HPO_4, 4 mM of EGTA, pH8, 15 mM of $MgSO_4$, 1 mM DDT, 1 mM ATP) in a Bertheld lumate (20 mM luciferin), wherein

A is infection with Bac-CMV-luci,

B is infection with BAC-PP-luci, and

1 is the activity of the luciferase per 10^4 cells.

Description of the invention

It is an object of the invention to construct a vector, which targets tissue cells, suitably liver cells highly specifically, *in vitro* and *in vivo*, is effectively

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taken up by the cells, and can direct the therapeutic genes into the cell nucleus. The vector is useful for gene therapy in various mammalian hosts.

The present invention comprises a tissue-specific vector, suitably a liver-specific vector for gene therapy of an animal host wherein an insect virus, suitably a baculovirus, or a nuclear polyhedrosis virus, which contains (i) a therapeutic DNA sequence, (ii) a promoter for gene expression in the target tissue, suitably the liver, and optionally (iii) an establishment sequence.

Although the present invention is described principally with reference to the liver-specificity of the vector, it is to be understood that the present invention encompasses mammalian animal tissue specificity more generally.

Baculoviruses belong to a family of large DNA viruses, the host spectrum of which is naturally limited exclusively to arthropods. Its genome (80 kbp - 200 kbp) is packed in a flexible nucleocapsid which permits the insertion of large amounts of foreign DNA. When it was recognized that the use of chemical insecticides is potentially dangerous to people and the environment, the pronounced host specificity of the baculoviruses suggested their use for biological insect control.

It is a prerequisite for this use of baculoviruses that the baculovirus gene is not expressed in human cells. It was observed in investigations of numerous human cell types that the treatment with baculoviruses did not result in any significant infections.

The cDNA of a gene, which is defective, absent or was changed by mutation in the case of the disease to be treated, is used as therapeutic DNA sequence for the vector of the present invention. Examples of such genes are the LDL receptor gene, the gene for the alpha-1-antitrypsin, for the blood clotting factors VIII and IX, for erythropoietin, and for thymidine kinase. A portion of a genomic sequence can also be used which bridges a mutation in the target gene, and can be recombined substantially homologously with this mutation.

Strong viral promoters, preferably the very early promoters of the cytomegalovirus (CMV), can suitably be used as promoters. Suitable liver-specific promoters, such as promoters/enhancers of the hepatitis B virus (HBV), such as the combination of core promoter/enhancer II, can also be advantageously employed. In addition to their specificity, they are also sufficiently small so that they can be easily incorporated into an expression vector. Promoters of liver-

specific genes, such as albumin, phosphoenol pyruvate carboxykinase (PEPCK), or ornithine transcarbamylase (OTC) can also be suitably employed.

The optional establishment sequence has the task of ensuring the stabilization of the vector in the cell without integration in the genome. It is used particularly in those cases where expression is necessary over prolonged periods. Suitable establishment sequences in accordance with the present invention include viral nucleus establishment sequences, such as those of the Epstein-Barr virus, or autonomous replication sequences from the mammalian genome.

The new vectors are essentially prepared by (a) cloning the therapeutic DNA sequence together with the promoter in a recombination vector, (b) optionally inserting an establishment sequence before or after the cloning, (c) transfecting the construct obtained together with the DNA of an insect virus into insect cells, and (d) obtaining from the supernatant of the insect culture the vector, packaged in the insect cells.

The present invention thus provides a novel vector for tissue-specific, suitably liver-specific gene transfer. This new vector offers appreciable advantages over previously developed viral vectors based on retroviruses or adenoviruses.

These advantages include tissue-specificity, suitably liver-specificity, the almost unlimited possibility for incorporating foreign DNA, the infection of cells incapable of dividing, the absence of cytotoxicity, and the simple generation of high-titer recombinant viruses.

As will be readily apparent to skilled practitioners, the vector of the present invention can be administered to the host, in any suitable manner known *per se*, in a suitable dosage form for the desired method.

It is particularly surprising that the vector infects particularly hepatocytes highly specifically. The vector makes possible the introduction of a desired gene into the liver of a patient and optimally configures the path of this gene to the site of functioning, because, for example, the vector is produced and administered to a patient through the bloodstream, suitably through the portal vein of the liver, or through the alimentary system. A significant prerequisite is thus fulfilled for the treatment of genetic diseases of the liver.

The present invention is described in greater detail by reference to the following specific example.

Example

Baculovirus Vector for the Expression of Luciferase in Hepatocytes

1. Construction of the baculovirus transfer vector

The *Pvu*II fragment of the pCMV-Luci, described by Mueller et al., in Proc. Natl. Acad. Sci. U.S.A. vol. 91, p. 2945 (1994) contains the cytomegalovirus promoter, the luciferase reporter gene and the CMV polyadenylation signal as the expression cassette. It is cloned into the *Sma*I restriction site of the pVL 1392. The resulting (pVL-CMV-Luci), purified by a Qiagen column*, is used to prepare a recombinant baculovirus.

The luciferase gene is obtained through *Bam*HI-*Hind* III restriction digestion and filling up the end by Klenow enzyme from the plasmid T7-lol. After cloning in the *Sma*I restriction site of the pVL-1392, the luciferase gene is under the control of the polyhedrin promoter (pVL-PP-Luci). This construct serves as a negative control, to illustrate the effect of the mammalian promoter in the case of the hepatocyte infection.

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The luciferase gene is replaced in an analogous construction by therapeutic genes, such as the LDL receptor gene. A liver-specific mammalian promoter (hepatitis) can be used instead of the CMV promoter. The polyhedrin sequences, flanking the expression cassette, can also be replaced by other nonessential baculovirus sequences, such as P94.

2. Preparation of recombinant viruses

pVL-CMC-Luci or pVL-PP-Luci (3 μg) and 0.5 μg of baculovirus wild type DNA (Baculo-Gold* PharMingen) are cotransfected by means of lipofectin (BRL) in 2 million *Spodoptera frugiperda* insect cells (Sf9, ATCC). Due to homologous recombination, brought about by the polyhedrin sequences of the transfer vectors, recombinant baculoviruses will result (Bac-CMV-Luci, Bac-PP-Luci), which carry the luciferase gene under the control of the CMV, or polyhedrin promoter.

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3. Reproduction and purification of the recombinant baculoviruses

Sf9 insect cells are infected in a bottle or spinner culture with an MOI of 1 for 3 days with the Bac-CMV-Luci or Bac-PP-Luci. The viruses formed are separated from the insect cells by centrifugation and subsequently pelletized at 12,000 g for 45 minutes. A band of purified virus is obtained by sucrose density gradient centrifugation for 4 hours at 100,000 g and with a gradient of 24% to 62%. This is transferred by pelletizing twice into the application buffer (isotonic salt solution or salt culture medium).

4. Investigations of the specificity of the infection or the luciferase expression of the CMV-Luci in liver cells

Mammalian cell lines of different tissues and species and primary human (phH) as well as primary murine (pmH) hepatocytes are infected with Bac-CMV-Luci or Bac-PP-Luci (MOI 100) to investigate the cell specificity of the baculovirus infection. The luciferase expression is measured after 1.5 days and the results show the specificity of the Bac-CMV-Luci in relation to the hepatocyte infection. Under control of the polyhedrin promoter (b: Bac-PP-Luci infection of different cell lines), the luciferase gene is active exclusively in insect cells.

5. Indications of a protein receptor-induced endocytosis of the baculovirus in hepatocytes (Huh7)

A protease treatment of the hepatocytes decreases the number of protein receptors, so that there is a reduction in the luciferase activity after Bac-CMV-Luci infection.

Enzyme	Concentration	% Reduction in Luciferase Activity
Trypsin	2 mg/mL	50.4
Papain	1 mg/mL	36.9
Pronase E	1 mg/mL	52.6

The foregoing table shows that Huh7 cells were treated for 15 minutes with the proteases specified. After the proteases were inactivated, the cells were infected for 1 hour with Bac-CMV-Luci (MOI 100). After the 1 hour virus infection, Huh7 cells were treated similarly for 3 hours (reference value: 100%). The reduction in the luciferase activity by a measurement after 1.5 days, arises out of the ratio of the luciferase value of the hepatocytes previously treated with protease to the reference value.

Chloroquine and colchicine intervene in the endocytosis pathway of the virus or in its migration into the hepatocyte nucleus. The luciferase activity is also decreased by the administration of either chemical.

	Concentration	% Reduction of Luciferase Activity
Chloroquine	0.5 mM	100
	0.1 mM	98.4
	0.03 mM	92
	0.01 mM	81
Colchicine	18 mg/mL	69.4
	6 mg/mL	56
	2 mg/mL	55

As shown in the foregoing table, Huh7 cells were infected with BAc-CMV-Luci (MOI 100), with chloroquine or colchicine being administered at the same time. The reduction in the luciferase activity is related to a reference value, for which the two chemicals were given in each case 12 hours after the infection. The expression of the luciferase was measured after 1.5 days.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A tissue specific vector for gene therapy of a host having a diseased liver, comprising an insect virus that contains (*i*) a therapeutic DNA sequence, (*ii*) a promoter for gene expression in the liver of the host, and optionally (*iii*) an establishment sequence.

2. The tissue specific vector of claim 1, wherein said insect virus is a baculovirus, or a nuclear polyhedrosis virus.

3. The vector of claim 1, wherein said therapeutic DNA sequence is the cDNA sequence of a gene that is defective, absent, or was changed by mutation by the disease to be treated.

4. The vector of claim 1, wherein said therapeutic DNA sequence comprises a portion of a genomic sequence from a gene which is not defective, bridging a mutation in a corresponding defective gene, and which can be recombined substantially homologously therewith.

5. The vector of claim 1, wherein said therapeutic DNA sequence comprises cDNA for the LDL receptor gene, the gene for alpha-1-antitrypsin, blood clotting factors VIII and IX, erythropoietin, or for thymidine kinase.

6. The vector of claim 1, wherein the promoter is a strong viral promoter.

7. The vector of claim 6, wherein said strong viral promoter is the very early promoter of the CMV, or a liver-specific promoter.

8. The vector of claim 7, wherein said liver-specific gene promoter is derived from HBV, or a combination of core-promoter/enhancer II.

9. The vector of claim 7, wherein said liver-specific gene promoter is albumin, PEPCK, or OTC promoter.

10. The vector of claim 1, wherein said establishment sequence is a viral nucleus establishment sequence.

11. The vector of claim 10, wherein said viral nucleus establishment sequence is derived from the Epstein-Barr virus, or an autonomous replication sequence.

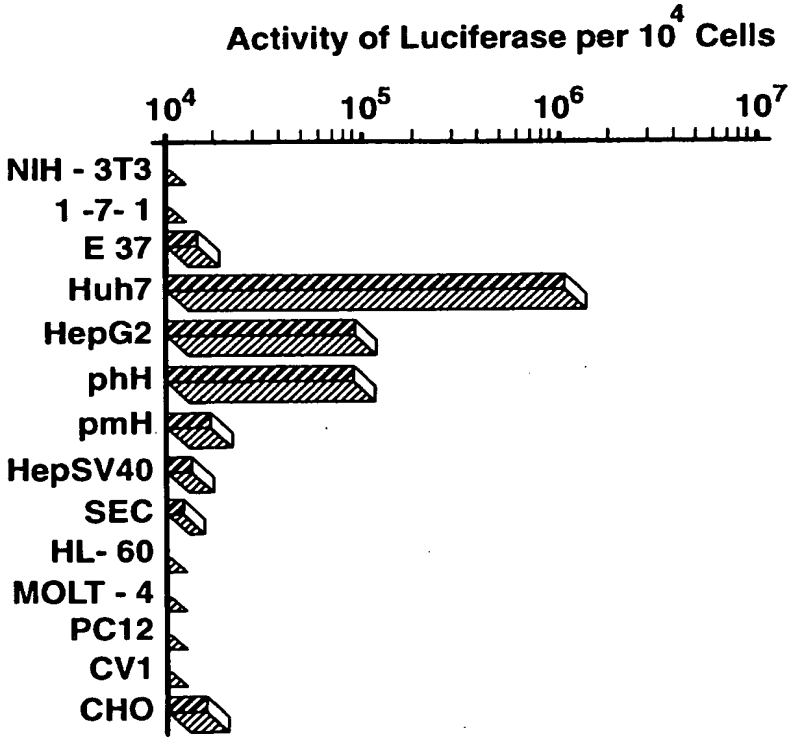
12. A method for producing the vector of claim 1, which comprises (*a*) cloning said therapeutic DNA sequence with said promoter in a recombination vector to obtain a construct, (*b*) optionally inserting said establishment sequence before or after said cloning, (*c*) transfecting said construct with the DNA of an insect virus into insect cells, and (*d*) obtaining from the supernatant of the insect culture the vector packaged in the insect cells.

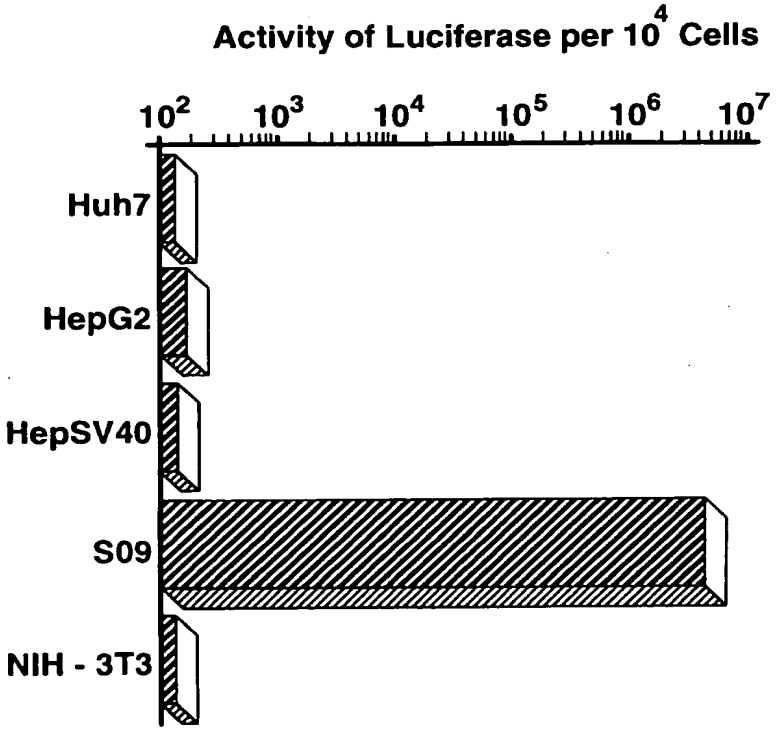
13. The method of claim 12, further comprising converting the vector into a dosage form that is suitable for administration to the host.

14. Use of the vector prepared by the method of claim 13 delivered intravenously for correcting a genetic defect of the liver.

15. Use of the vector according to claim 14, wherein said promoter is derived from HBV and the vector is delivered directly to the liver through the portal vein of the liver of the host.

16. Use of the vector prepared by the method of claim 13 for correcting a genetic defect of the liver, wherein the vector is absorbed by the alimentary system of the host.


Activity of Luciferase per 10^4 Cells
10^4
10^5
10^6
10^7
NIH - 3T3
1 -7- 1
E 37
Huh7
HepG2
phH
pmH
HepSV40
SEC
HL- 60
MOLT - 4
PC12
CV1
CHO


Activity of Luciferase per 10^4 Cells
10^2
10^3
10^4
10^5
10^6
10^7
Huh7
HepG2
HepSV40
S09
NIH - 3T3